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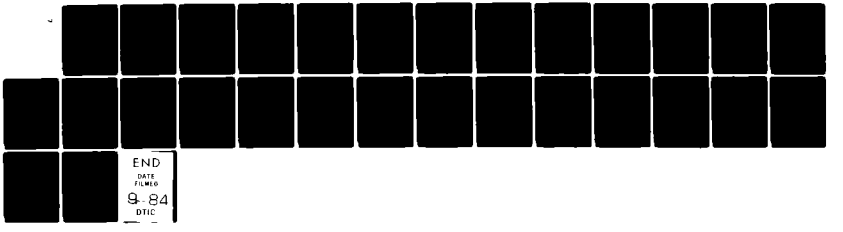

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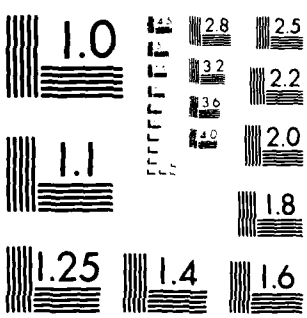

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20. The dielectric behaviour of water and aqueous solutions of myoglobin, DNA and human serum low-density lipoprotein (LDL) has been investigated over a wide frequency range. By combining the measured complex permittivity of pure water at frequencies up to 70GHz with literature values in the far infrared it is shown that the dielectric behaviour of water may be characterised by a small subsidiary dispersion centred around 600GHz, in addition to the well known microwave dispersion. The value of the infinite frequency permittivity in respect of this principal dispersion region was found to be 5.74±0.31 at 20°C.

Aqueous solutions of various forms of DNA were investigated between 2-18GHz but no dielectric behaviour was observed which could not be explained by classical dielectric theory. The interpretation of the dielectric measurements on aqueous solutions of myoglobin and LDL shows that both types of molecule, despite the large disparity in their size, attract a layer of irrotationally bound water of average width 1-2 molecules. Using this fact a mathematical model of a hydrated macromolecule in a continuum was set up and the microwave energy deposition was calculated and compared with that of bulk water. It is shown that the specific energy absorption rate (SAR) in bound water may be substantially higher than the SAR in bulk water at frequencies above a half a decade on either side of 1GHz. The precise frequency and the value of the energy enhancement ratio depends upon the ionic conductivity and upon the value assumed for the relaxation frequency of the bound water. It is shown that the value of this parameter for myoglobin bound water is around 4 GHz which is rather higher than had hitherto been assumed.

Determination of bound water in biological tissue and energy dissipated in bound water by low level microwaves

1. Introduction

The purpose of this research was to investigate the quantity and nature of bound water in biological material using dielectric methods. Having characterised the bound water by knowledge of its dielectric properties the energy dissipation may be calculated. Apart from this, however, the bound water dielectric parameters are of value in the field of molecular biophysics, the essential structure and conformation of biological molecules being strongly dependent upon the characteristics of the bound water shell. In the present programme of work dielectric measurements were carried out on aqueous solutions of myoglobin, low-density lipoprotein (LDL) and DNA. In addition a study of the dielectric behaviour of pure water at frequencies up to 70GHz was made in the hope that the acquisition of extra knowledge at millimetre wavelengths would provide extra facts about its idspersion behaviour. On the theoretical side, a model was set up to enable the specific energy absorption in bound water to be calculated and compared with that in bulk water. Some of the results of the work have already been described in previous reports (Progress reports, Physiology Program, Office of Naval Research 1979, 1980; ONR-AIBS Meeting, December 1981) and in original publications (Dawkins, Nightingale, South, Sheppard and Grant 1979, Nightingale, Szwarnowski, Sheppard and Grant 1981, Grant, Szwarnowski and Sheppard 1981, Grant, Szwarnowski and Sheppard 1981, Grant, Nightingale, Sheppard and Gough 1981, Grant 1982). Therefore this report will concentrate on the more recent

work (some still to be published) and upon general implications.

Dielectric techniques have been used to study bound water in biological material since the 1930's. The principle of the method is that water molecules adjacent to a biological macromolecule have, in order to rotate in an electric field, to transcend a higher potential barrier than that experienced by water molecules in the bulk of the liquid. This is, of course, directly due to the stronger forces acting upon the water molecules neighbouring the macromolecule. Therefore these molecules will possess a longer rotational correlation time than the bulk water molecules which, in turn, will mean that the dielectric dispersion region occurs at a lower frequency. The term 'bound' water must be understood in this context; other definitions can equally well be produced according to the experimental technique chosen for its study. In all cases it can however be assumed that solute-water bonds will be broken less often than the water-water bonds in the bulk liquid. Many authorities prefer the term 'water of hydration' to 'bound water' and the two expressions will be used interchangeably in this report.

The presence of a separate dielectric dispersion region due to water of hydration was proposed first by Schwan (1957,1965) in respect of his measurements on haemoglobin, and verified later for the albumins (Grant 1965,1966). In both sets of investigation it was proposed that the dielectric relaxation frequency of the bound water should occur at a few hundred megahertz, in contrast to around 20GHz where the bulk water in protein solutions had been shown to relax (Buchanan, Haggis, Hasted and Robinson 1952, Grant 1957). Hence the bound water dispersion in a protein solution is at frequencies between the bulk water and the solute dispersions, the latter occurring at around 1MHz (Oncley 1943). From knowledge of the dielectric parameters of the bound water a suitable model may be set up in order

to calculate the energy deposition in an assembly of hydrated macromolecules exposed to electromagnetic radiation. This formed the first part of the current project.

2. Absorption of microwave energy by bulk water and water of hydration

The deposition of microwave (300MHz-300GHz) energy within biological material is determined largely by the amount of water and dissolved ions present. Cellular and sub-cellular structures and polar biological macromolecules also contribute to the absorption but are of less significance at these, rather than at lower, frequencies. Over certain parts of this frequency range the bound water may be expected to absorb energy more strongly than the free water owing to its different dielectric properties. In order to calculate the magnitudeof this differential absorption two models were set up and appropriate calculations made.

In the first model the biological macromolecule was assumed to be spherical and of radius 5nm surrounded by a uniform layer of bound water of width 0.6nm, although the theory is valid up to radii of at least 10^{-5}m. A plane electromagnetic wave was considered to be incident upon such a sphere immersed in an infinite dielectric medium. The average power dissipation (P_D) per unit volume of hydration shell was calculated (Dawkins et al.1979) by evaluating the integral

$$P_D = \frac{3\,(\sigma + \epsilon_0 \omega \epsilon'')}{8\pi\,(b^3 - a^3)} \int E \cdot E^* \, dv \qquad (1)$$

using the integral relations between the associated Legendre polynomials

given by Stratton (1941). In this equation σ and ϵ'' are the ionic conductivity and the dielectric loss factor respectively, b is the radius of the hydrated particle and a is that of the anhydrous particle. E is the electric field and E* its complex conjugate. The angular frequency ω is 2 X the frequency of the incident radiation. The calculations were carried out for various values of ionic conductivity σ and using values of the dielectric loss (ϵ'') of bound water calculated according to four assumed relaxation frequencies (f_R); 158, 500, 1580 and 5000MHz. The ratio of the energy deposition per unit volume of bound water to that of free water (the enhancement factor F) was then calculated for various combinations of σ and f_R. For values of conductivity of 0.001 Sm^{-1} for both the bound water (σ_B) and the bulk water (σ_w) the magnitude of F was shown to bein excess of 20 at frequencies at the lower end of the microwave region ($\sim$300MHz). For more biologically realistic values of σ (1.0Sm^{-1}) the highest value of F was found to be around 2.5 and occurred near 1GHz for an assumed value of $f_R \simeq$ 500MHz. By ascribing different values of σ to the bound and bulk water the size of F can be further varied and would become very high if $\sigma_B > \sigma_w$.

This simple model of a hydrated molecule was extended to cater for the case where the bound water shell is not uniform but which has dielectric properties varying across it (McClean et al.1981). The procedure adopted was to divide the shell into five sub-shells each with a different relaxation frequency. A modified version of equation (1) was computed using spherical Bessel and Hankel functions, and then solved for various combinations of σ_w, σ_B and f_R, as previously. The model was then extended still further (McClean et al.1981) to take in the case of ten sub-shells. The results of the calculations agreed broadly with those obtained in respect of the more simple model, involving a uniform shell for the water of hydration.

The energy deposition per unit time per unit volume (P_D) calculated from equation (1) is of course directly proportional to the specific absorption rate (SAR), so it is instructive to consider the implications of the present calculations regarding the biological effects of microwaves. Bound water by its very definition means water molecules strongly coupled to the biological macromolecule. Thereby any excess deposition of energy in the bound water shell might reasonably be expected to give rise to more energy absorption by the macromolecule than would be the case if these water molecules were uniformly distributed throughout the bulk of the tissue. The familiar idea of the motion of three bodies coupled by two springs of differing Young's moduli might be a useful analogy. However a clear understanding of how energy is communicated over distances of a few molecular diameters is not yet available, and therefore proposals concerning localised effects must remain speculative. On the other hand conclusions arising from the present work in respect of bulk energy deposition in bound water are rigorous and mean that at certain frequencies the energy dissipated by microwave absorption in biological tissue is much greater if the water present is in the bound rather than the free state. Such calculated values of energy absorption and resulting temperature rise will therefore be seriously underestimated for certain tissues if all the water is assumed to behave like free water. An example of such a tissue is lens material where between a quarter and a half of the water content is bound water. the actual value depending upon whether the estimation is carried out using biochemical methods (Rink 1978) or physical techniques (Dawkins, Gabriel, Sheppard and Grant 1981).

3. Influence in biological macromolecules on the dielectric behaviour of water

In previous work information on water structure in biological solution using dielectric methods has been gained either by studying the effect of the water molecules on the solute dispersion, or by trying to isolate the effect of the bound water and investigating its own dispersion (the δ -dispersion). These methods have been explained in full previously (Grant, Sheppard and South 1978). Much less attention has been paid to the possibility of characterising the total water dispersion in the biological solution and then comparing its behaviour with that of pure water, the dielectric parameters of the latter being well known up to frequencies of several tens of GHz. To carry out this kind of investigation successfully it is necessary to have available equipment which will measure at frequencies higher than the relaxation frequency of pure water. This is one reason why there has been in the past a dearth of measurements on the dielectric properties of biolgical solutions in the region of the water dispersion, although the situation has been redressed recently by Foster, Schepps and Epstain (1982).

In the present work dielectric measurements were made on aqueous solutions of myoglobin and low-density lipoprotein (LDL), these being examples of small and large biological molecules respectively. The molecular weight of myoglobin is 17000 and that of LDL is in excess of 10^6. Measurements were also made on the dielectric properties of DNA to check the interesting observation by Swicord and Davis (1983) that aqueous solutions of DNA exhibit enhanced absorption in the 8-12GHz frequency region.

3 (a) Dielectric behaviour of pure water

In order to make effective comparison between the dielectric behaviour of a biological solution with that of pure water it is assumed that the dielectric parameters of the latter are known accurately. While this is true in respect of the static permittivity (ϵ_s) and the relaxation time (τ) there is still considerable ignorance concerning the value of the infinite frequency permittivity (ϵ_∞), and there are large deficiences in knowledge of the general dielectric behaviour of water between around 50GHz and the far infrared region. It was therefore decided to measure the complex permittivity of water at 21 different frequency points between 1.9-15GHz, plus a point at 70GHz (Nightingale et al.1981, Grant et al.1981a), and to then combine the data with those of Afşar and Hasted (1977) taken in the frequency range 200GHz-11THz. To complete the set of data a point at 35GHz taken from the work of Grant and Shack (1967) was also included. The total set of data used for the analysis consisted of 56 frequency points in the range 1.8GHz-11THz, the temperature of measurement being 20°C.

Ignoring resonance effects which occur at frequencies in excess of 2THz it was found that the data could be fitted to the expression

$$\hat{\epsilon} = \epsilon' - j\epsilon'' = n^2 + \frac{\epsilon_s - \epsilon_\infty}{1 + j\omega\tau_1} + \frac{\epsilon_\infty - n^2}{1 + j\omega\tau_2} \qquad (2)$$

where n is the refractive index of water at frequencies in the region of 2THz, τ_1 is the relaxation time appropriate to the familiar microwave dispersion and τ_2 is the relaxation time of a process taking place in the far infrared. The other symbols have their usual significance.

Table 1 Dielectric parameters of pure water at 20°C

	Parameter	Value	95% Confidence Interval
Principal Dispersion	ϵ_s	80.1	-
	τ_1 (ps)	9.48	0.07
Subsidiary Dispersion	ϵ_{∞}	5.74	0.31
	τ_2 (ps)	0.25	0.08
	n^2	3.34	0.38

The results of the fit are shown in Table 1, from which several important conclusions follow. The first is that the value of ϵ_{∞} for the microwave dispersion is 5.74 at 20°C with an associated error (the 95% confidence limit) only a little greater than 5%. This is now the most accurate value available in the literature for ϵ_{∞}. It should be noted, however, that the relative permittivity ϵ' never actually reaches a plateau in this frequency region; in fact ϵ' diminishes continuously with frequency from microwave frequencies right up to near 7THz, when it starts to increase owing to resonance absorption. Thus ϵ_{∞} can not be measured directly and is of limited physical significance. It should be regarded as an important mathematical parameter occurring in the Debye equation which is itself an accurate description of the dielectric properties of water up to frequencies of around 50GHz. The second important revelation from Table 1 is that a new dispersion region has been shown to exist centred

around a relaxation frequency near 600GHz (0.25ps). The change in relative permittivity of 2.40 throughout the dispersion is small but significant. The origin of the dispersion at a molecular level must remain speculative but one possibility would be to ascribe it to the rotation of the zero bonded and one bonded molecules present in pure water. It was proposed by Haggis, Hasted and Buchanan (1952) that water could be considered as a statistical assembly of molecules making 4,3,2,1 and 0 bonds. In that case the zero bonded and single bonded molecules would be able to rotate in an electric field without breaking a hydrogen bond and should therefore exhibit their dispersion at frequencies higher than the principal dispersion, which is known to occur in the microwave region. The calculated contribution to the relative permittivity from the zero and one bonded molecules is 1.7, which agrees satisfactorily with the observed 2.4 considerin experimental error (Table 1) and the limitations of the model. The value of the relative permittivity at the high frequency end of this dispersion ($\epsilon' = n^2 = 3.34$) is of course greater than the square of the optical refractive index of water ($n_\infty = 1.69$), owing to presence of further dispersion in the infrared and at higher frequencies.

3 (b) Dielectric properties od aqueous solutions of DNA

Although the principal objective of this programme was to investigate the behaviour of water in biological solutions using dielectric methods, it was also decided to measure the dielectric properties of aqueous solutions of DNA for their own sake. The main reason for undertaking this work was because Swicord and Davis (1983) had reported an unusually high degree of attenuation in aqueous solution of DNA extracted from E.Coli irradiated by microwaves in the frequency region 8-12GHz. They later extended the work to enzyme activated DNA and found an even more enhanced absorption. On the other hand no absorption effects were noticed for solutions of the commercially available DNA salt

which could not be explained by classical dielectric theory.

In the present work the following DNA solutions were investigated, all at a concentration of 1% : (1) Calf thymus DNA sodium salt (Sigma Chemical Company), (2) Sonicated calf thymus sodium salt DNA, (3) pure E.Coli DNA (Sigma Chemical Company), (4) enzyme treated calf thymus DNA, (5) enzyme treated E.Coli DNA. Samples (1) and (2) were measured at 22 different frequencies in the range 2-18GHz, this range being chosen in order to cover adequately the region investigated by Swicord and Davis (8 GHz) and give an ample overlap at each end. Sample (1) was measured at 5,10,20,25 and 30°C and sample (2) at 10°C. Samples (3) and (4) and (5) were measured at 9.5GHz and 25°C. The enzyme used was DNAase (Sigma Chemical Company) and was added to the DNA sample in a proportion of 0.1%. Measurements of ε' and ε'' were made immediately after the addition of the enzyme and then at 10 minute intervals up to 100 minutes, followed by further measurements approximately every 30 minutes up to six hours. The DNA solutions were prepared by dissolving the solute in water and adding Tris buffer to control the pH at 7.0.

Table 2 Dielectric parameters of 1% aqueous solution sodium salt DNA (calf thymus)

Temperature °C	Dielectric Increment (Δ)	Relaxation Frequency f_R(GHz)	Cole-Cole Distribution Parameter (α)
5	80.0±0.2	10.54±0.05	0.04±0.04
10	76.4±0.4	12.66±0.09	0.01±0.01
20	74.8±0.2	16.56±0.10	0.03±0.01
25	71.7±0.2	19.42±0.11	0.01±0.01
30	71.0±0.2	21.96±0.13	0.03±0.01

The results for sample (1) are shown in Table 2. Comparison of the values of these data with those of the equivalent parameters for pure water provides the following facts. The value of Δ for the 1% DNA solution is on average about 1% lower than for pure water, as would be expected using simple volume proportion considerations. The relaxation frequencies in Table 2 do not differ from those of pure water to within experimental error, which again accords with expectation for a dilute solution. The value of the Cole-Cole distribution parameter α appears to be a little greater than that for pure water but the effect is barely significant. The parameters were obtained by fixing the value of ϵ_∞ at the value of 5.74, obtained as described in Section 3(a) of this report. The values of ϵ' and ϵ'' obtained for sonicated DNA (Sample 2) agreed to within experimental error with those measured for Sample 1 and gave dielectric dispersion parameters similar to those shown in Table 2. Since Sample 1 was highly polymerised high molecular weight DNA it is clear that breaking up the DNA molecule into fragments by the sonication

procedure has no effect on the dielectric properties of the solution at frequencies appropriate to the water dispersion.

Particular interest centres around the dielectric behaviour of Sample (3) because it was the DNA extracted from E.Coli which Swicord and Davis found gave an attenuation coefficient in aqueous solution some 40% higher than pure water at 8GHz and 10-12% higher at 12GHz. In contrast our dielectric measurements taken over the frequency range 7.5-14GHz showed no differences from those obtained from samples 1 and 2, and gave dispersion parameters which agreed to within experimental error with those in Table 2. Experimental attention was particularly focussed on a frequency of 9.48GHz and a temperature of 25°C. Values obtained for the 1% solution of E.Coli DNA in three independent experiments were $\epsilon' = 61.9\pm0.5$, $\epsilon'' = 28.5\pm0.4$; $\epsilon' = 62.4\pm0.3$, $\epsilon'' = 28.4\pm0.3$; $\epsilon' = 62.4\pm0.4$, $\epsilon'' = 28.2\pm0.3$. The mean values may be written $\underline{\epsilon' = 62.2\pm0.2}$, $\underline{\epsilon'' = 28.3\pm0.2}$. The corresponding mean values for the three experiments on the sodium salt DNA were $\epsilon' = 62.1\pm0.2$, $\epsilon'' = 28.4\pm0.2$. Thus there is no evidence for any differences between the dielectric behaviour or the absorption characteristics between the two samples.

The next question concerns enzyme treatment, Swicord and Davis observing increases in the attenuation coefficient as high as 70% greater than water after the addition of the enzyme. In the present work the dielectric properties as a function of time after the addition of the enzyme were investigated for both the E.Coli DNA and the sodium salt DNA, but no variations were observed up to a time of three hours. Measurements were performed at 9.45GHz and at 25°C but for both samples ϵ' remained between 49.7 and 50.3 and ϵ'' between 31.7 and 32.0 over the entire three hour period. Readings were taken at 20 different times starting at 10 minute intervals and finishing with 30 minute intervals when it became clear that there were no changes.

In conclusion it appears that the present programme of work does not support the existence of any aspects of dielectric behaviour in aqueous solutions of DNA which can not be explained by classical dielectric relaxation theory. However because the possibility of any such existence of resonance absorption in DNA at microwave frequencies has such important implications, both in respect of basic science and microwave radiation hazards, it is important that it should be studied further, particularly as there is theoretical support for its occurrence (Van Zandt, Kohli and Prohofsky 1982). This importance is re-enforced by the discovery of an unexpected and as yet unexplained dispersion in aqueous DNA solution in the 10-1000MHz frequency region (Takashima, Gabriel, Sheppard and Grant 1984).

3 (c) Dielectric properties of water in aqueous solutions of low-density lipoprotein (LDL) and myoglobin

As mentioned in the Introduction and described in full elsewhere (Grant et al.1978) dielectric methods for investigating the behaviour of water in biological material have been in existence for half a century. There has been intense activity in this area over the past decade, particularly in respect of dielectric measurements on solid tissues where knowledge of the electrical properties is necessary to the full understanding of the hazards of non-ionising radiation and the use of radiowaves and microwaves in cancer therapy. There has been rather less work recently on the dielectric behaviour of biological solutions, particularly at frequencies in the neighbourhood of the water relaxation, and yet it is more possible to interpret unambiguously the dielectric data and to identify the appropriate mechanism for an aqueous solution than for a complicated tissue. Accordingly the present programme was arranged to study aqueous solutions of LDL and myoglobin and thus to compare the behaviour of large abd moderately sized

biological molecules on their water environment.

LDL is usually defined as that group of lipoproteins having a density range 1.006-1.063 and a flotation rate of 0-20 svedberg units. Flotation rate is equivalent to velocity/acceleration, and 1 Svedberg unit equals 10^{-13}S. In human plasma LDL is the most abundant lipoprotein and is normally present in a concentration of about 350mg/100ml of plasma. The LDL molecule is ideal for a dielectric study in that it has roughly equal numbers of phospholipids and glycopeptides which are themselves approximately equally divided between being positively and negatively charged. Moreover the molecule is almost exactly spherical, with a radius of 11nm. The protein moiety is on the outside of the molecule and therefore may be expected to exhibit similar forces in respect of the surrounding water molecules as a globular protein. In contrast the myoglobin molecule is much smaller, having an effective radius of 1.8nm. The molecule is not spherical but approximates to a prolate spheroid of axial ratio 2:1, but such a shape exhibits dielectric behaviour indistinguishable from that of a sphere. Therefore when comparing and contrasting the dielectric behaviour of LDL and myoglobin in water the significantly different parameter is the molecular size, other factors being of minimal importance.

The LDL was isolated from human volunteers and the samples were prepared by Dr. M.J.Chapman of the INSERM Research Unit on the Metabolism of Lipids at the Hopital Henri Mondor, Paris. This collaboration was part of a research programme funded by the NATO Scientific Affairs Division. The sample preparation was as described previously (Chana, Chapman, Sheppard, Mills, Goldstein and Grant 1980). Measurements of $\hat{\epsilon} = \epsilon' - j\epsilon''$ were made at 15 frequencies in the range 2-18GHz, at a temperature of 10°C and at a concentration of 109mg/ml. Two further samples (86 and 41 mg/ml) were prepared by dilution and the

measurements repeated under the same frequency and temperature conditions. These samples will be referred to as 1,2 and 3 respectively. The best fit to the data was found to be a Cole-Cole dispersion and the fitted parameters are shown in Table 3.

Table 3 Dielectric behaviour of low-density lipoprotein solution at 10°C

Concentration (mg/ml)	Dielectric Increment (Δ)	Relaxation Frequency f_R (GHz)	Cole-Cole Distribution Parameter (α)
109	66.51±0.47	11.88±0.14	0.04±0.01
86	68.84±0.37	12.02±0.11	0.04±0.01
41	73.93±0.04	12.37±0.095	0.03±0.01

(f_R for pure water at 10°C is 12.47GHz)

The high frequency permittivity ϵ_∞ was clamped at the water value (Section 3a) this being regarded as the best available approximation. Considering sample 1 the value of ϵ' obtained by adding Δ to ϵ_∞(5.74) is found to be 72.25 as compared with the static permittivity of pure water which is 83.83. It has been shown previously (Grant, Sheppard, Mills and Slack 1972) that the hydration (W) of LDL can be represented to a good approximation by

$$c(\bar{v} + W) = 9(\epsilon_w - \epsilon_L) \qquad (3)$$

where C is the concentration expressed in mg/ml, $\bar{v}$ is the partial specific volume of the LDL, ϵ_L is the relative permittivity of the LDL solution at around 800MHz and ϵ_w is that of water at the samr frequency. Assuming that the partial specific volume is 0.97 and is known to an accuracy of 2%, and allowing for experimental error in ϵ_L the maximum value of hydration is calculated as 0.05g/g. This figure is much lower than has been obtained for small globular proteins, and at first sight may appear to be in contradiction. However if the width of the shell of bound water required to provide a weight fraction of 0.05 is calculated a different picture emerges. Assuming a radius r for the LDL molecule and a width dr for the hydration shell then

$$\frac{\frac{4}{3}\pi(r+dr)^3 - \frac{4}{3}\pi r^3}{\frac{4}{3}\pi r^3 \rho} = 0.05 \qquad (4)$$

where ρ is the density of the LDL, i.e. the reciprocal of $\bar{v}$. Since r = 11nm the calculation gives dr= 0.19nm which is rather more than the radius of one water molecule. Thus the average width of the hydration shell does not depart appreciably from the value of 1-2 layers of water molecules well accepted in respect of globular proteins. The ratio (R) of bound to bulk water in the solution can be calculated from the expression

$$R = \frac{\left(1+\frac{dr}{r}\right)^3 - 1}{1/c - 1} \qquad (5)$$

where C is the volume concentration of the LDL. Substituting in to equation (3) gives R=0.6%. Thus the proportion of bound water present in the solution is negligible even though the concentration of the LDL is more than 10%. This clearly makes the point that it is

meaningless to quote the proportion of bound to bulk water in a biological solution unless the nature of the solute molecule is specified.

Another interesting observation from Table 3 is that the relaxation frequency is reduced by a few percent from the value for pure water (12.5GHz); in addition the value of α is significantly greater than zero. These two facts indicate that the presence of the LDL does affect the dielectric properties of the bulk water even though the proportion of irrotationally bound water is small. The explanation of this is not clear unless there are some long range forces present as has been suggested previously to account for the saturation of the dielectric increment of the α dispersion of concentration above 30mg/ml (Essex, Grant, Sheppard, South, Symonds, Mills and Slack 1977).

The myoglobin work can be summarised as follows. Previous published work within this research programme (Grant et al.1981b) confirmed the existence of a β -dispersion with a relaxation frequency of around 2MHz, and caused by the rotation of the myoglobin molecule as a rigid spheroid. More recently the frequency region 100MHz-18GHz has been concentrated upon. Myoglobin solutions of concentration 170mg/ml and pH = 6.4 have been investigated at five temperatures - 9, 15, 25, 35 and 45°C. The samples measured were sperm whale myoglobin from Miles Laboratories (PTY) Limited and the myoglobin was dissolved and de-ionised water without the necessity of further purification. The complex permittivity $\epsilon' - j\epsilon''$ was measured at 44 different frequencies, making a total of 88 data points in the frequency range 100MHz - 18GHz (fig.1). The measurements on ϵ' were extended down to 100kHz in order to assist data analysis, although

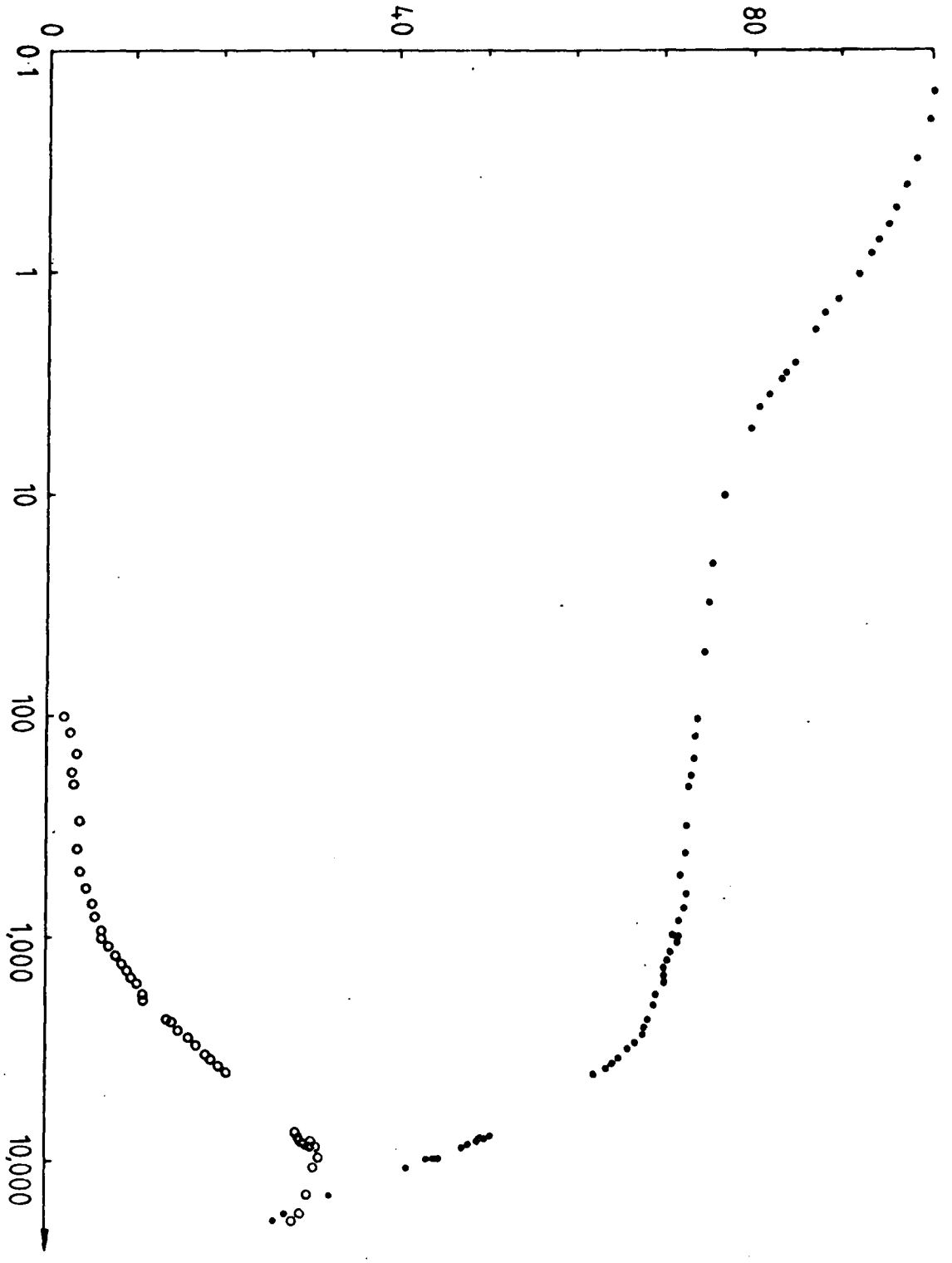

Fig.1 Complex permittivity of aqueous solution of myoglobin (concentration 170mg/ml; temperature 25°C)

• • • .... ϵ'

o o o .... ϵ''

Frequency in MHz

in the present programme attention is focussed on the water relaxation i.e. the main interest is at frequencies of around 1GHz and above. Analysis of the data shows that the dielectric behaviour of the water can be represented as a Cole-Cole dispersion with a relaxation frequency at each temperature slightly lower than that for pure water.

Table 4 Dielectric behaviour of water in myoglobin solution of concentration 170mg/ml. Single dispersion fit

Temperature °C	Dielectric Increment (Δ)	Relaxation Frequency ζ_R(GHz)	Cole-Cole Distribution parameter (α)
9	67.4±0.3	11.3±0.1	0.06±0.01
15	66.4±0.2	13.5±0.1	0.06±0.01
25	63.1±0.2	17.7±0.2	0.06±0.01
35	60.2±0.2	23.3±0.2	0.05±0.01
45	57.6±0.2	29.2±0.3	0.07±0.01

The parameter values are shown in Table 4 and it is seen that the general characteristics of the dielectric behaviour are similar to those observed for solutions of DNA and LDL. However, with the myoglobin much more experimental information was available and other fits to the data may be attempted. Applying equation (5) to the 17% myoglobin solution and assuming that the hydration layer is 0.35nm in width (South and Grant 1972) the value of R is calculated as around 10%. Hence 90% of the water present is not irrotationally

bound. Even if generous allowance is made for error due to the limitations of the model, or to the possible existence of long range forces, it seems clear that a substantial fraction of the water present should behave as pure water. Accordingly an attempt was made to replace the single dispersion representation in Table 4 by two dispersions, one of which has its relaxation frequency at each temperature clamped at a value equal to that of pure water. The root mean square error of the fit was comparable with that obtained in the single dispersion fit and therefore it is an acceptable alternative. The dielectric parameters of this fit are shown in Table 5.

Table 5 Dielectric behaviour of water in myoglobin solution of concentration 170mg/ml. Double Debye dispersion fit Dispersion having same properties as pure water

Temperature °C	Dielectric Increment (Δ)	Relaxation Frequency f_R, (GHz)
9	62.0±0.7	12.1
15	60.5±0.9	14.5
25	57.6±0.9	19.2
35	57.0±0.3	24.3
45	55.0±0.3	29.6
	Extra water dispersion	
9	5.0±0.6	2.9±0.6
15	5.6±0.8	3.7±0.7
25	5.4±0.8	4.0±0.8
35	3.4±0.3	4.1±0.7
45	2.9±0.3	4.1±0.7

Comparison of the dielectric increments between Tables 4 and 5 show that the value of Δ at each temperature in Table 4 is equal to the sum of the increments in Table 5. It therefore appears as though Table 4 represents the average dielectric behaviour of water as a whole whereas the interpretation in Table 5 is dividing the water into two categories. If the smaller of the two dispersions can be equated with the water of hydration, and assuming that the quantity of water in each category is roughly proportional to the magnitude of respective increment (Δ), then the ratio of bound water to total water is around 8-9% i.e. in good agreement with the value calculated above on geometric considerations. Moreover if the quantity of bound water is calculated from the amplitude Δ_2, using the mixture relationship as described by Grant, Mitton, South and Sheppard (1974), a value of bound water of 0.25g/g of myoglobin is obtained. This again corresponds to a hydration layer 1-2 molecules in width, which therefore strengthens further the evidence that this dispersion is due to the water of hydration. The value of the relaxation frequency (3-4GHz) is higher than has been observed previously for most proteins although Pennock and Schwan (1969) proposed for haemoglobin that f_R should be placed in the region 0.5-1GHz, which is not too disimilar from the present findings. It is interesting in this respect that haemoglobin and myoglobin have the same side groups.

Further evidence for the existence of bound water relaxation at these frequencies comes from the work of Epstein, Foster and Mackay (1983) who observed a relaxation frequency of 3-4GHz for water of hydration associated with microemulsions. Finally, recent work (as yet unpublished) in our laboratory on various tissues has revealed

a dispersion region at frequencies of a few GHz and it is unlikely that this could be due to anything other than bound water. Phenomena such as the Maxwell-Wagner effect, side chain rotation (Pennock and Schwan 1969) and proton fluctuation (South and Grant 1973) effects are very unlikely to occur at frequencies in excess of 1GHz.

4. General Conclusions

The dielectric properties of water in solutions of myoglobin and low-density lipoprotein (LDL) indicate that, despite the large disparity in the size of the macromolecule, 1-2 layers of irrotationally bound water (water of hydration) exist on the surface of the macromolecule. This, therefore, is the fundamental parameter which should be quoted when characterising bound water. In contrast the weight fraction of water of hydration per unit mass of solute varies widely from one sort of macromolecule to another. The relaxation frequency of the bound water also varies according to the solution or tissue but evidence is growing that it occurs at frequencies a little above 1GHz rather than a factor of two to five below. Relatively speaking, this means that the differences between bound and bulk water are less distinct, and the differential energy absorption between the two types of water is a little reduced.

Measurements made at frequencies of 2-18GHz on the dielectric properties of aqueous solutions of various types of DNA, including enzyme activated solutions, indicate no behaviour which can not be interpreted by classical dielectric theory. It would however be worthwhile to investigate further the mechanisms of interaction of radiowaves and microwaves by extending the frequency range.

The dielectric behaviour of pure water indicates the presence of a small dispersion region centred around 600GHz. This means that the dielectric properties of water at frequencies in excess of a few tens of GHz have a contribution from this dispersion in addition to the contribution from the well established microwave dispersion. It also follows that the widely used infinite frequency permittivity (ϵ_∞), hitherto regarded as a fundamental parameter of the microwave dispersion, does not correspond to a plateau in the relative permittivity. It is of course still a useful mathematical parameter for analysing data taken at the low frequency end of the microwave region and in this respect the improvement in the accuracy by which ϵ_∞ is known (5.74±0.31 at 20°C) is a useful gain.

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